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Biomarkers in WNT1 and PLS3 osteoporosis: Altered concentrations of DKK1 and FGF23

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Supplemental data are included.

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29 **Disclosures**

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31 interest.

Abstract

Recent advancements in genetic research have uncovered new forms of monogenic osteoporosis, expanding our understanding of the molecular pathways regulating bone health. Despite active research, knowledge on the pathomechanisms, disease-specific biomarkers and optimal treatment in these disorders is still limited. Mutations in *WNT1*, encoding a WNT/β-catenin pathway ligand WNT1, and *PLS3*, encoding X chromosomally inherited plastin 3 (PLS3), both result in early-onset osteoporosis with prevalent fractures and disrupted bone metabolism. However, despite marked skeletal pathology, conventional bone markers are usually normal in both diseases. Our study aimed to identify novel bone markers in PLS3 and WNT1 osteoporosis that could offer diagnostic potential and shed light on the mechanisms behind these skeletal pathologies. We measured several parameters of bone metabolism, including serum dickkopf-1 (DKK1), sclerostin, and intact and C-terminal fibroblast growth factor 23 (FGF23) concentrations in 17 *WNT1* and 14 *PLS3* mutation-positive subjects. Findings were compared with 34 healthy mutation-negative subjects from the same families. Results confirmed normal concentrations of conventional metabolic bone markers in both groups. DKK1 concentrations were significantly elevated in *PLS3* mutation-positive subjects compared with *WNT1* mutation-positive subjects ($p<0.001$) or the mutation-negative subjects ($p=0.002$). Similar differences were not seen in WNT1 subjects. Sclerostin concentrations did not differ between any groups. Both intact and C-terminal FGF23 were significantly elevated in *WNT1* mutation-positive subjects ($p=0.039$ and 0.027, respectively) and normal in *PLS3* subjects. Our results indicate a link between PLS3 and DKK1 and WNT1 and FGF23 in bone metabolism. The normal sclerostin and DKK1 levels in patients with impaired WNT signaling suggest another parallel regulatory mechanism. These findings provide novel information on the molecular networks in

bone. Extended studies are needed to investigate whether these biomarkers offer diagnostic value or potential as treatment targets in osteoporosis.

Key words: WNT signaling, PLS3, dickkopf-1, sclerostin, fibroblast growth factor 23, osteoporosis

Introduction

The discovery of new forms of monogenic osteoporosis has brought an abundance of new knowledge on the molecular pathways and specific proteins participating in bone health maintenance. In 2013, we and others showed that WNT1 is a key ligand to the WNT pathway in bone as monoallelic and biallelic mutations in *WNT1* were identified to cause severe and early-onset autosomal dominant osteoporosis and autosomal recessive osteogenesis imperfecta, respectively[1]. The mutated WNT1 leads to low activation of the WNT pathway, decreased expression of target genes and consequently low bone turnover, low bone mineral density (BMD) and prevalent fractures[1]. Similarly, in 2013, mutations in Plastin 3–encoding *PLS3*, were reported to result in X-linked childhood-onset osteoporosis with frequent peripheral and vertebral compression fractures and low bone turnover with heterogenous and defective mineralization in bone biopsies[2–5]. Due to its X-chromosomal inheritance pattern, the phenotype is typically more severe in affected males, while females have normal to increased skeletal fragility[2,3].

The molecular mechanisms by which WNT1 and PLS3 modulate bone metabolism are very different. WNT signaling regulates bone cell development and differentiation directly and is a crucial component of skeletal development and homeostasis from early fetal development all throughout childhood growth and adulthood maintenance[6]. Its aberrant activation has previously been demonstrated in several monogenic bone diseases with severe skeletal pathology; mutations in the transmembrane co-receptor low-density lipoprotein receptor-related protein 5 (LRP5) lead to osteoporosis-pseudoglioma syndrome and high bone mass disorder[7], and mutations in *SOST*, encoding WNT-pathway inhibitor sclerostin, result in sclerosing bone diseases sclerosteosis and van Buchem disease[8,9]. Furthermore, genome-wide association studies have highlighted the pathway's

role in bone health and the pathway is currently recognized as a preferable target for novel osteoporosis drugs[10,11]. The functions of PLS3 in bone metabolism, on the other hand, are still largely unidentified. Animal studies indicate a role in regulation of cytoskeletal actin bundling[12] and their mechanosensory apparatus[2], and studies on patients' bone biopsies suggest involvement in osteoclast function as well as bone matrix mineralization[5]. However, the exact functions of PLS3 and the pathways which its actions diverge with remain elusive.

Vertebral fractures are a common feature in both WNT1 and PLS3 osteoporosis while the incidence of peripheral fractures varies. DXA-derived BMD values range from normal to severely reduced depending on patient age, gender and type of mutation[1,3–5]. Furthermore, despite the distinct skeletal pathologies, conventional metabolic bone markers have been reported to be normal in affected *WNT1* and *PLS3* mutation-positive subjects[1,3,13,14]. This great variability in clinical presentation often complicates and delays diagnosis. Therefore, we set out to evaluate, in addition to the conventional bone turnover markers, the circulating concentrations of dickkopf-1 (DKK1; dickkopf WNT signaling pathway inhibitor 1), sclerostin, and fibroblast growth factor 23 (FGF23) in *WNT1* and *PLS3* mutation-positive subjects to identify potential biomarkers for these two bone diseases and to further elucidate the molecular mechanisms behind their disturbed bone metabolism. All three markers—DKK1, sclerostin and FGF23—are mainly secreted by the osteocytes. Sclerostin and DKK1 are known inhibitors of WNT signaling in bone and target molecules for novel osteoporosis drugs, namely anti-sclerostin and anti-DKK1 antibodies[11,15]. FGF23 is a hormone partaking in the regulation of serum phosphate concentration through renal excretion and intestinal absorption[16] although additional functions have also been suggested in e.g. iron metabolism, inflammation and erythropoiesis[17,18]. Furthermore, we have previously reported altered osteocyte protein expression in bone biopsies of patients with WNT1 and PLS3

osteoporosis[19]. Here, we report intriguing and counterintuitive findings of significantly elevated and gender-dependent concentrations of DKK1 in *PLS3* mutation-positive subjects and normal concentrations in *WNT1* mutation-positive subjects, and significantly elevated FGF23 concentrations in *WNT1* mutation-positive subjects.

Patients and methods

Subjects

We recruited *WNT1* mutation-positive subjects from two large Finnish families (Family A and B) with the same heterozygous missense mutation p.C218G in *WNT1* as reported elsewhere[1,13,14]. For the present study, we offered participation to all previously identified mutation-positive subjects (n=25). A control group, with similar genetic background and representing all age groups and both genders, was formed by offering participation to mutation-negative individuals in these two families (n=32). Altogether, 17 mutation-positive and 17 mutation-negative individuals consented from these two families.

We recruited *PLS3* mutation-positive subjects from four previously identified Finnish families with different *PLS3* mutations[3,4,17]: Family C = an intronic splice site mutation c.73-24T>A (p.Asp25Alafs*17)[3], Family D = a 12.5 kb tandem duplication spanning intron 2 to 3 of *PLS3*[20], Family E = a nonsense mutation c.766C>T (p.Arg256*)[4], and Family F = a *de novo* heterozygous missense mutation c.1424A>G (p.N446S)[4]. We offered participation to all mutation-positive individuals (n=14, n=3, n=2, n=1, respectively) in these four families. Similarly, participation was also offered to mutation-negative individuals from the same four families. Altogether, 14 mutation-positive and 17 mutation-negative individuals consented.

Upon participation, all subjects signed a written informed consent to participation in the study. All genetic and clinical studies were approved by the Research Ethics Board of Helsinki University Hospital.

Genetic evaluations

We have previously, in our prior studies on monogenic forms of osteoporosis, identified the same *WNT1* mutation in two unrelated Finnish families[1,13] and four different *PLS3* mutations in four Finnish families[3,4,20]. These were identified using different methods, including targeted Sanger sequencing, whole-genome sequencing, and custom-made array-comparative genomic hybridization ([1,3,4,13,20]). In the current study, we screened all participating study subjects for the pertinent gene mutation with conventional Sanger sequencing on DNA extracted from peripheral blood as previously described[13].

Clinical assessments

We gathered data on previous fractures and prior or current osteoporosis and other medications by patient questionnaires and from hospital records. BMD measures were collected from previously performed dual-energy X-ray absorptiometry–assessments, which were all performed at different time points and using different machines. Measured BMD values are given as Z-scores, calculated using equipment-specific normative data; these are used to roughly compare and differentiate between normal and osteoporotic BMD status.

Biochemical evaluations

We collected all blood samples in the morning between 8 and 9 a.m. after an overnight fast. Serum aliquots were immediately stored at –80°C until analyses. Serum concentrations of ionized calcium, phosphate and alkaline phosphatase (ALP), and urinary phosphate and creatinine were analyzed by routine clinical laboratory assays at the HUSLAB Laboratory, Helsinki, Finland. Serum 25-hydroxyvitamin D concentrations were determined with a chemiluminescent immunoassay (CLIA) on two analyzers: the Architect i2000SR analyzer (Abbott, Deerfield, IL, USA) with an assay performance of: analytical range 10–300 nmol/L, intra-assay coefficient of variation (CV) of <6%, and interassay CV <8%; and the Advia Centaur XPT analyzer (Siemens, Raritan, NJ, USA) with an assay performance of: analytical range 10.5–375 nmol/L, intra-assay CV of <6%, and interassay CV <9%. Serum 1,25-dihydroxyvitamin D was analyzed by CLIA on a LIAISON XL analyzer (DiaSorin, Stillwater, MN, USA) with an assay performance of: analytical range 12–480 pmol/L, intra-assay CV of <4%, and interassay CV <5%. Both parathyroid hormone (PTH) and collagen type 1 cross-linked C-telopeptide (CTX; a bone resorption marker) were assessed with CLIA assays on the IDS-iSYS fully automated immunoassay system (Immunodiagnostic Systems, Ltd., Bolton, UK). The PTH assay performance was as follows: analytical range 5–5000 pg/mL, intra-assay CV of <4%, and interassay CV <5%. The CTX assay performance was as follows: analytical range 0.033–6.000 ng/mL, intra-assay CV of <6%, and interassay CV <10%. Serum intact FGF23 was determined by an enzyme-linked immunosorbent assay (ELISA) (Kainos Laboratories, Inc., Tokyo, Japan) with an assay performance of: analytical range 8–800 pg/mL, intra-assay CV of <6%, and interassay CV of <10%. Serum C-terminal FGF23 was assessed by ELISA (Biomedica, Vienna, Austria) with an assay performance of: analytical range 0.1–20.0 pmol/L, intra-assay CV of <12%, and interassay CV <10%. Serum DKK1 was measured by ELISA (Biomedica) with an assay performance of: analytical range 1.7–160 pmol/L, intra-assay CV of <3%, and interassay CV <5%. Serum sclerostin was determined by ELISA (Biomedica) with an assay performance of: analytical range 3.2–240 pmol/L, intra-assay CV of <7%,

and interassay CV <10%. Serum type I procollagen intact N-terminal propeptide (PINP) was assessed with the UniQ radioimmunoassay (Orion Diagnostica, Espoo, Finland) with an assay performance of: analytical range 5–250 µg/L, intra-assay CV of <5%, and interassay CV <6%. All samples were run in duplicates and in full accordance with the manufacturers' instructions for all biochemical assays.

Statistical analyses

Descriptive data are reported as median and range when appropriate. Normality of data was assessed by the Kolmogorov–Smirnov and Shapiro–Wilk tests, and visually using histograms. Unpaired two-tailed Student's *t* test, Mann–Whitney *U* test, and Pearson correlation were used as appropriate (SPSS Statistics 24; IBM Corporation, Armond, NY, USA). *p*-values <0.05 were considered statistically significant.

Results

Subjects

The current study comprised a total of 17 *WNT1* mutation-positive subjects (12 females, age range 11–76 years, median 52 years) from two families with a *WNT1* missense mutation p.C218G (Families A and B) and 14 *PLS3* mutation-positive subjects (9 females, 8–76 years, median 41 years) from four families with different *PLS3* mutations (Families C–F) (Figure 1, Table 1). The control subjects consisted of altogether 34 mutation-negative individuals (17 females, 8–77 years, median 36 years) from these same six families (Figure 1, Supplemental Table 1).

The mutation-positive subjects had varying histories of previous fractures (range 0 to >10 fractures), vertebral compression fractures and osteoporosis medications (Figure 1, Table 1). Treatment for

osteoporosis was ongoing for three subjects at the time of study. Another three subjects received inhaled glucocorticoid treatment for asthma with low to moderate dosages. The mutation-negative subjects also had varying histories of previous fractures (range of peripheral fractures 0–9) but none had ongoing osteoporosis medication at the time of the study (Supplemental Table 1). Six of the subjects had ongoing glucocorticoid treatment for asthma; all were in low to moderate dosages and given in inhaled form.

Biochemical markers of bone and mineral metabolism

Evaluations of serum ionized calcium, phosphate, 1,25-dihydroxy- and 25-hydroxyvitamin D and PTH, and urinary phosphate concentrations showed mostly normal and only isolated findings of supra- and subnormal values in both mutation-positive and mutation-negative subjects; no differences between the groups were noted (Figure 2, Supplemental Table 2). Similarly, no differences between the groups were observed for the bone turnover markers PINP, ALP and CTX (Figure 2, Supplemental Table 2).

DKK1, sclerostin and FGF23 concentrations

We observed no significant differences in serum DKK1 between the *WNT1* mutation-positive (median 27.3 pmol/L; range 13.2–58.9 pmol/L) and the mutation-negative (median 27.9 pmol/L; 4.4–81.8 pmol/L) subjects (p=0.583) (Figure 3, Supplemental Table 2). Correspondingly, the serum sclerostin concentrations were similar between the *WNT1* mutation-positive and the mutation-negative subjects: 19.5 pmol/L (8.9–34.0 pmol/L) and 19.8 pmol/L (4.3–123 pmol/L), respectively (p=0.905) (Figure 3, Supplemental Table 2).

On the contrary, both C-terminal and intact FGF23 concentrations were significantly elevated in the *WNT1* mutation-positive subjects compared with the mutation-negative subjects (medians 1.51 vs 0.96 pmol/L and 54.9 vs 51.4 pg/mL; p=0.027 and p=0.039, respectively) (Figure 4, Supplemental Table 2). Of note, despite elevated FGF23, serum and urinary phosphate concentrations were similar between the mutation-positive and negative subjects (Supplemental Table 2).

We found DKK1 to be significantly elevated in the *PLS3* mutation-positive subjects in comparison with the *WNT1* mutation-positive subjects (medians 27.3 pmol/L (13.2–58.9 pmol/L vs 53.3 pmol/L, p<0.001) and mutation-negative subjects (27.9 pmol/L (4.4–81.8 pmol/L), p=0.002) (Figure 3, Supplemental Table 2). Post-hoc analysis by gender confirmed that female *PLS3* mutation-positive subjects (n=9) had significantly increased DKK1 concentrations in comparison with female mutation-negative subjects (n=17) (p=0.009) (Figure 3). The difference for *PLS3* mutation-positive males (n=5) vs mutation-negative males (n=17) was less distinct (p=0.100) (Figure 3). The DKK1 concentrations in the *PLS3* mutation-positive subjects did not vary depending on age (r=0.192; p=0.510) nor depending on past or ongoing osteoporosis medication (Figure 2). Sclerostin concentrations did not differ between the *PLS3* mutation-positive and the mutation-negative subjects: 17.4 pmol/L (5.0–43.0 pmol/L) vs 19.8 pmol/L (4.3–123.0 pmol/L) (p=1.000) (Figure 3). No significant differences were noted in FGF23 concentrations between the *PLS3* mutation-positive and the mutation-negative subjects (p=0.610 for C-terminal and p=0.634 for intact) (Figure 4).

Discussion

This study is the first to systematically assess several conventional and new bone markers, including DKK1, sclerostin, and intact and C-terminal FGF23, in a large cohort of pediatric and adult subjects

249 with *WNT1* or *PLS3* mutations. Mutations in *WNT1* and *PLS3* are known to cause severe, early-onset
250 osteoporosis with frequent fractures, low BMD, and on the tissue level, low bone turnover and
251 distinct bone pathology[1,3,4,13]. While the skeletal consequences of aberrant WNT1 signaling are
252 quite well understood, the primary pathways and molecular mechanisms by which abnormal PLS3
253 function results in skeletal disease are still largely unknown. Additionally, recognizing the inaccuracy
254 of conventional metabolic bone markers in evaluating bone health in osteoporotic patients[21] and
255 their normality in low-turnover, monogenic and collagen-independent skeletal disorders[1,3,13,14],
256 evaluating alternate biomarkers offers novel information and potential targets for future diagnostic
257 and therapeutic means. We report novel findings suggesting a link between PLS3 and DKK1 in bone
258 metabolism with an increase in serum DKK1. In addition, we report normality of DKK1 and sclerostin
259 in WNT1-related bone disease, but elevated serum intact and C-terminal FGF23 in *WNT1* mutation-
260 positive subjects. These findings shed light on possible pathomechanisms behind these skeletal
261 disorders and on the key proteins governing bone health.
262
263 WNT pathway is a key regulator of skeletal development from early fetal period to all throughout
264 childhood, adolescent growth and mature bone homeostasis in adulthood and its aberrant
265 activation leads to several skeletal disorders of both low and high bone mass[7–9]. DKK1, encoded by
266 *DKK1*, is an inhibitor of the WNT signaling pathway and thus an important factor maintaining
267 balanced bone metabolism. Its primary source in bone is presumably osteocytes and the role to
268 inhibit WNT signaling by binding to the transmembrane dual-receptor complex consisting of LRP5
269 or LRP6 and seven transmembrane G-protein Frizzled and inactivating this receptor complex[6,22,23].
270 Sclerostin, encoded by *SOST* and also secreted by osteocytes, acts in a similar manner by targeting
271 LRP5/6 and reducing binding of a WNT ligand to the receptor complex[6]. WNT signaling is bone
272 formation–favoring as it promotes first mesenchymal progenitor cell commitment to the

273 osteoblastic lineage and then osteoblast differentiation, proliferation and activity. In normal
274 conditions, WNT signaling and its inhibition by DKK1 and sclerostin are kept at refined balance to
275 maintain sufficient and to detain excessive bone formation. Serum concentrations of sclerostin are
276 reported to vary between different age groups and genders and also largely depending on the
277 method used for analysis[24,25,26,27]. For DKK1, on the other hand, information about its association
278 with age or gender is limited and from the few studies reported, no association was observed.
279
280 The heterozygous *WNT1* mutation p.C218G leads to decreased activation of the WNT pathway, low
281 target gene transcription and consequently low bone formation and turnover[1]. DKK1 and sclerostin
282 function to inhibit WNT signaling and in the presence of decreased WNT pathway activation due to
283 the mutated WNT1, one would hypothesize that this would lead to decreased concentrations of the
284 pathway's inhibitors and that concentrations of DKK1 and sclerostin would be subsequently similarly
285 decreased. The finding of unaltered circulating DKK1 and sclerostin in *WNT1* mutation-positive
286 subjects is unexpected and suggests that no compensatory feed-back mechanisms exist from the
287 intracellular WNT/β-catenin activity to its negative regulators. This particular *WNT1* mutation only
288 leads to haploinsufficiency and slightly reduced WNT1 signaling which may impact the results[1].
289 Other plausible explanations could be that the actions by DKK1 and sclerostin are outplayed by other
290 partaking or compensatory mechanisms, or that the communication between WNT1, sclerostin and
291 DKK1 is not as exclusive as previously thought[21].
292
293 On the contrary, we observed significantly elevated serum concentrations of FGF23 but normal
294 phosphate parameters in *WNT1* mutation-positive subjects. This is congruent to our previous
295 findings of high expression of FGF23 in bone biopsies from *WNT1* mutation-positive subjects[19]. The
296 FGF23 concentrations did not vary depending on age or gender, as supported by general

297 assumptions that serum FGF23 concentrations do not associate with age, gender or puberty[28].
298 Furthermore, we did not find significant differences in serum phosphate or vitamin D concentrations
299 between the groups. FGF23 is mainly secreted from osteocytes, regulates primarily systemic
300 phosphate homeostasis and its malfunction is linked to several inherited syndromes with
301 hypophosphatemic rickets and tumor-induced osteomalacia[29]. However, the impact of FGF23 on
302 serum phosphate levels in normal physiological and osteoporotic conditions is not well described.
303 We and others have previously reported that neither intact nor C-terminal FGF23 levels correlate
304 with serum or urinary phosphate in children, suggesting additional roles for FGF23 in bone
305 metabolism[30,31]. This might be reflected in the *WNT1* subjects in our study, where additional
306 mechanisms are likely to be included. The link between FGF23 and WNT1 in bone is unclear and we
307 have previously postulated that increased FGF23 in response to low WNT signaling—independent
308 of changes in PTH—could be mediated by altered nuclear receptor–associated protein 1[24,32].
309 However, given our previous findings of low bone marrow iron storage[27] and the previously
310 identified link between iron metabolism and FGF23[33,34], the rise in circulating FGF23 could be in
311 result of an iron-deficient microenvironment in bone. However, these interactions between
312 different proteins are likely very complex and demand further functional investigations.
313
314 Although PLS3 has an inevitably important role in bone metabolism, as demonstrated by the skeletal
315 phenotypes in *PLS3* mutation-positive patients, the molecular mechanisms behind the grave
316 skeletal changes are still largely unknown. PLS3 has been shown to modulate actin bundling and
317 cytoskeletal remodeling and is thought to enable cell endo- and exocytosis, migration and
318 adhesion[34,35]. In bone specifically, PLS3 was first suggested to be involved in osteocytes'
319 mechanosensing abilities[2,12], and, more recently, experimental findings suggest involvement in
320 osteoclastogenesis and osteoclast function through impaired podosome organization[36,37].

Evaluations of patients' bone biopsies collectively insinuate a mineralization defect, which could stem from a combination of these different mechanisms[5,23,39].

The finding that mutations in *PLS3* somehow relay to altered DKK1 secretion is novel and important. Our group has previously reported delayed and disturbed bone matrix mineralization in a young male with a *PLS3* deletion[5], but the mechanism behind PLS3's presumed role in matrix mineralization has remained elusive. Further, although WNT signaling and one of its antagonists DKK1, are important for bone metabolism and DKK1 has been shown to inhibit matrix mineralization in a dose-dependent manner[6,39], the mechanism by which defective PLS3 leads to altered DKK1 concentrations is unclear. One plausible explanation could be that the mutated PLS3 affects osteocyte function, leading to increased DKK1 production and secretion. Saupe *et al.* have previously described that drug-induced disruptions in actin cytoskeleton and focal adhesion signaling impacted *DKK1* mRNA levels in tumor cells[40]. The effects of *PLS3* mutations on DKK1 production could also be indirect, transmitted through other, yet unidentified proteins. Further, the reason for low bone turnover in PLS3 osteoporosis could reside in defective WNT signaling, which might also explain the similarities in phenotype between *WNT1* and *PLS3* mutation-positive patients. Our post-hoc analysis by gender confirmed the main finding in female patients. Although DKK1 was elevated also in *PLS3* mutation-positive males compared to healthy subjects the post-hoc analysis did not reach statistical significance, probably due to lack of statistical power based on the low number of male patients (n=5). Possible gender differences remain unclear and unanswered by our study and demand further investigation. Furthermore, if the changes in DKK1 are due to cytoskeletal changes and osteocyte dysfunction, it remains unclear why sclerostin concentrations remain unaltered. Lastly, the normality of the osteocyte-derived FGF23 in PLS3 subjects is also an interesting and important observation.

Both DKK1 and sclerostin are target molecules for novel osteoporosis treatments; anti-DKK1 and anti-sclerostin antibodies are to counteract these proteins' inhibitory actions and enhance WNT-driven bone formation[11,15]. While promising in postmenopausal osteoporosis treatment[11,15], their therapeutic efficiency in WNT1- or PLS3-related bone diseases are yet to be verified. For WNT1 osteoporosis, the unexpected normality in sclerostin concentrations could indicate that anti-sclerostin antibody might be a very effective treatment modality for *WNT1* mutation-positive subjects. Since the low WNT signaling in *WNT1* mutation-positive subjects is not corrected by feedback regulation to subsequently reduce the negative effect of DKK1 and sclerostin, it is possible that their inhibitory effect on the WNT pathway amplifies the effect of absent WNT stimulus on bone metabolism, further reducing bone formation and contributing to the skeletal phenotype. Moreover, the relatively high concentrations of DKK1 and sclerostin could partly explain the severe bone phenotype in heterozygote *WNT1* subjects. Correspondingly, the surprising finding of elevated DKK1 in *PLS3* mutation-positive subjects might imply that this could similarly be a suitable route for effective treatment in PLS3 osteoporosis. Lastly, with novel anti-FGF23 antibodies providing positive findings in preliminary mouse studies and clinical trials[41], similar approaches could be taken to evaluate to efficiency of blocking FGF23 signaling to enhance bone quality in WNT1-related bone disorders.

While conventional markers of bone turnover are typically informative when monitoring treatment response, their value in evaluating altered turnover in individual patients is limited, even in high-turnover cases[24]. In *WNT1* and *PLS3* mutation-positive subjects, the conventional metabolic markers are normal despite clear skeletal phenotypes and increased bone fragility[1,3,14,15]. One reason can be that recommended bone markers for clinical use, i.e. CTX and PINP[24], mainly reflect

turnover of collagen type I, while PLS3 and WNT1 osteoporosis are associated with collagen-independent, partly still unidentified biological mechanisms. In addition, both conditions are identified as low-turnover osteoporosis, whereby the sensitivity of conventional bone markers is an additional limitation for identifying alterations.

We acknowledge certain limitations in our study. These primarily concern the small cohort size of mutation-positive subjects and the lack of longitudinal assessment of how DKK1 and sclerostin concentrations might respond to disease progression or osteoporosis treatment. For this reason, we were not able to assess whether these biomarkers could be used as predictive markers of future fractures, which should be evaluated in future studies. We also were not able to fully evaluate possible correlation between the biomarker concentrations and BMD. Since the patients originated from various parts of the country, BMD assessments were performed using different DXA machines and therefore we were able to only use Z-values from equipment-specific normative data. Some study participants, among both the mutation-positive and mutation-negative subjects, received inhaled glucocorticoid treatment at the time of the study. None of these subjects, however, were treated with high dosages or with oral glucocorticoids. Since the impact of inhaled glucocorticoids was regarded minimal[42], we did not exclude them from the study. Furthermore, the selection of the mutation-negative individuals for the control group was based solely on family relations, in order to have groups with similar overall genetic backgrounds, while disregarding age, gender or menopausal status as inclusion or exclusion criteria. Lastly, in the premise of this study, we were not able to further functionally evaluate for example bone tissue expression of DKK1 or sclerostin or the detailed communications between these proteins. The potential of improving bone health in these patients by targeting these molecules remains to be elucidated in future studies. Nonetheless, given the rarity of both *WNT1* and *PLS3* mutation-positive subjects, a control group of individuals

393 from the same families and the novelty of the research topic, we consider our results to be highly
394 valuable and provide unique and novel information on the molecular mechanisms behind these
395 monogenic skeletal pathologies.
396
397 In conclusion, our results intriguingly indicate increased DKK1 concentration in PLS3 osteoporosis
398 and suggest a link between PLS3 and DKK1 in bone metabolism. Sclerostin concentrations are
399 normal in WNT1 and PLS3 osteoporosis but FGF23 may be impacted by abnormal WNT1 signaling.
400 These findings provide novel information on the molecular communications in bone and open up
401 new avenues for focused studies on mechanisms in PLS3- and WNT1-related skeletal disorders.
402 DKK1 and FGF23 may be clinically useful biomarkers for PLS3 and WNT1 osteoporosis, respectively.
403 Future studies should investigate the relevance of these findings in larger patient cohorts and in
404 clinical treatment trials targeting WNT pathway antagonists.

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Author roles

Study design: REM, PM, OM. Study conduct: REM, PM, OM. Data collection: REM, PM. Data analysis: all authors. Drafting of the manuscript: all authors. Revising of the manuscript: all authors. Approving the final version of the manuscript: all authors.

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587 **Figures**

588

589 **Figure 1. Pedigrees of the six included Finnish families with WNT1 and PLS3 mutations**. A) and B)
590 *WNT1* missense mutation c.652T>G (p.C218G) C) *PLS3* intronic splice site mutation c.73–24T>A
591 (p.Asp25Alafs*17), D) intragenic tandem duplication within *PLS3*, E) *PLS3* de novo heterozygous
592 missense mutation c.1424AG> (p.N466S), and F) *PLS3* nonsense mutation c.766C>T (p.Arg256*). The
593 pedigrees have been modified to ensure anonymity. Squares represent males, circles females.

594

595 **Figure 2. Scatter plots of bone turnover marker serum concentrations in 17 *WNT1* mutation-**
596 **positive and 14 *PLS3* mutation-positive subjects and 34 mutation-negative subjects.** A)
597 Parathyroid hormone (PTH); B) Collagen type 1 cross-linked C-telopeptide (CTX-1); C) Alkaline
598 phosphatase (ALP); D) Type I procollagen intact N-terminal propeptide (PINP); E) Sclerostin; F)
599 Dickkoph-1 (DKK1); and fibroblast growth factor 23 (FGF23) in G) intact and H) C-terminal form. Blue
600 represents WNT1 subjects, red PLS3 subjects, gray circles mutation-negative subjects. The *WNT1*
601 mutation-positive subjects harbor a heterozygous *WNT1* missense mutation c.652T>G (p.C218G).
602 The *PLS3* mutation-positive subjects harbor different *PLS3* mutations: nine with an intronic splice
603 site mutation c.73–24T>A (p.Asp25Alafs*17), three with a duplication of exon 3, and two with a
604 nonsense mutation c.766C>T (p.Arg256*). Subjects with ongoing osteoporosis treatment at the
605 time of the study are indicated by black marker outlines.

606

607 **Figure 3. Box plots of serum DKK1 and sclerostin concentrations in 17 *WNT1* mutation-positive**
608 **subjects (A–B) and 14 *PLS3* mutation-positive subjects (C–D) compared with 34 healthy mutation-**
609 **negative subjects.** MP = mutation-positive, MN = mutation-negative, F = female, M = male. The *PLS3*
610 mutation-positive subjects harbor different mutations: seven with heterozygous and two with

611 hemizygous deletion c.73–24T>A (p.Asp25Alafs*17); one with heterozygous and two with
612 hemizygous duplication of exon 3; and one with heterozygous and one with hemizygous nonsense
613 mutation c.766C>T (p.Arg256*). All *WNT1* mutation-positive subjects harbor a heterozygous
614 missense mutation c.652T>G (p.C218G). For **B)** and **D)** the scale has been adjusted for visual clarity,
615 leaving one outlier (MN-34; 123 pmol/L) outside the graph. p-values derived from Mann–Whitney
616 U test.
617
618 **Figure 4. Box plots of serum intact and C-terminal FGF23 concentrations in 17 *WNT1* mutation-**
619 **positive subjects (A–B) and 14 *PLS3* mutation-positive subjects (C–D) compared with 34 healthy**
620 **mutation-negative subjects.** MP = mutation-positive, MN = mutation-negative, F = female, M =
621 male, I = intact, C = C-terminal. The *PLS3* mutation-positive subjects harbor different mutations:
622 seven with heterozygous and two with hemizygous deletion c.73–24T>A (p.Asp25Alafs*17); one
623 with heterozygous and two with hemizygous duplication of exon 3; and one with heterozygous and
624 one with hemizygous nonsense mutation c.766C>T (p.Arg256*). All *WNT1* mutation-positive
625 subjects harbor a heterozygous missense mutation c.652T>G (p.C218G). For **B)** the scale has been
626 adjusted for visual clarity, leaving one outlier (WNT1-2; 11.02 pmol/L) outside the graph. p-values
627 derived from Mann–Whitney U test.

Tables

Table 1. Clinical data for 17 *WNT1* and 14 *PLS3* mutation-positive subjects.

Subject, Family	Gender	Age (years)	Number of peripheral fractures	Vertebral compression fractures	Prior osteoporosis medication, years since last dose	Type of osteoporosis medication	Inhaled glucocorticoids**	Postmenopausal	BMD (Z-scores)		
									LS	Fem	WB
***WNT1* mutation-positive subjects**											
WNT1 mutation-positive subjects n=17; median age 52 years; 12 females/5 males; % of postmenopausal females (of all, of females) 35%, 75%											
WNT1 mutation-negative subjects n=17; median age 31 years; 8 females/9 males; % of postmenopausal females (of all, of females) 6%, 11%											
WNT1-1, A	F	11	1	No	No	None	No	No	-0.5	0.0	-0.5
WNT1-2, A	F	13	1	No	No	None	No	No	-2.1	-1.3	N/A
WNT1-4, A	F	17	9	No	Yes*	PAM	No	No	-2.5	-0.5	-1.0
WNT1-6, A	F	34	2	No	No	None	No	No	-1.5	-2.2	-2.2
WNT1-7, A	F	44	0	No	No	None	No	No	-1.4	-0.9	N/A
WNT1-8, A	F	48	>5	Yes	Yes, 3	ZOL	No	No	-1.2	-2.4	-2.0
WNT1-9, A	F	52	0	No	No	None	No	Yes	N/A	N/A	N/A
WNT1-11, A	F	53	5	Yes	Yes, 2	DMAB	No	Yes	-2.0	-1.0	-1.1
WNT1-12, A	F	54	0	No	No	None	No	Yes	-3.3	-1.1	-1.5
WNT1-14, B	F	68	1	Yes	No	None	No	Yes	0.2	-1.0	-1.5
WNT1-15, A	F	71	9	Yes	Yes, 12	EST, RIS	Yes	Yes	-1.5	-1.6	N/A
WNT1-16, A	F	74	6	Yes	Yes*	ALN, PTH, ZOL, DMAB	No	Yes	-0.8	-1.9	N/A
WNT1-3, A	M	13	4	No	No	None	No	N/A	-1.1	-0.5	-0.7
WNT1-5, A	M	19	2	No	No	None	No	N/A	-2.2	-0.1	N/A
WNT1-10, A	M	52	2	Yes	No	None	No	N/A	-2.8	-1.3	-2.3
WNT1-13, A	M	63	3	Yes	Yes, 5	ZOL, PTH	No	N/A	-2.2	-1.3	-3.5
WNT1-17, A	M	76	1	Yes	Yes, 6	ZOL, PTH	No	N/A	0.1	0.0	N/A
***PLS3* mutation-positive subjects**											

PLS3 mutation-positive subjects n=14; median age 41 years; 9 females/5 males; % of postmenopausal females (of all, of females) 21%, 33%											
PLS3 mutation-negative subjects n=17; median age 39 years; 9 females/8 males; % of postmenopausal females (of all, of females) 18%, 25%											
PLS3-2, C	F	11	0	No	No	None	No	No	N/A	N/A	N/A
PLS3-3, C	F	14	0	No	No	None	No	No	N/A	N/A	N/A
PLS3-4, C	F	15	0	No	No	None	Yes	No	-2.2	-1.2	-1.1
PLS3-7, D	F	41	0	Yes	No	None	Yes	No	N/A	N/A	N/A
PLS3-8, C	F	41	0	Yes	Yes*	ZOL	No	No	-2.2	-1.5	-2.0
PLS3-10, C	F	48	0	No	No	None	No	No	-0.6	0.5	-0.6
PLS3-11, C	F	51	0	Yes	Yes, 5	PTH	No	Yes	-1.3	N/A	-0.7
PLS3-12, E	F	57	1	No	No	None	No	Yes	1.2	1.1	-0.7
PLS3-13, C	F	69	>10	Yes	Yes, 4	PTH, ZOL	No	Yes	-2.3	-0.6	-1.9
PLS3-1, D	M	8	2•	Yes	No	None	No	N/A	N/A	N/A	N/A
PLS3-5, D	M	21	1	Yes	Yes, 9	ZOL	No	N/A	-0.3	-1.4	-0.2
PLS3-6, E	M	32	4	Yes	None	None	No	N/A	-4.1	-3.3	-4.5
PLS3-9, C	M	45	10•	Yes	Yes, 7	ZOL	No	N/A	-1.9	-1.8	-2.5
PLS3-14, C	M	76	4	Yes	Yes, 4	ZOL	No	N/A	-2.2	N/A	-2.3

Mutation-positive subjects from families A and B harbor a heterozygous *WNT1* missense mutation c.652T>G (p.C218G). Mutation-positive subjects from families C to F harbor different *PLS3* mutations: Family C = intronic splice site mutation c.73–24T>A (p.Asp25Alafs*17), Family D = duplication of exon 3, Family E = nonsense mutation c.766C>T (p.Arg256*). F = female, M = male, LS = lumbar spine, Fem = femoral neck, WB = whole body, ALN = alendronate, ZOL = zoledronic acid, PTH = teriparatide, DMAB = denosumab, EST = estrogen, RIS= risedronate, PAM = pamidronate.

• Last fracture within 12 months prior to the study

* Ongoing osteoporosis medication at the time of study

** Inhaled glucocorticoids with low to moderate dose; none of the subjects received oral glucocorticoids

Figure 1.

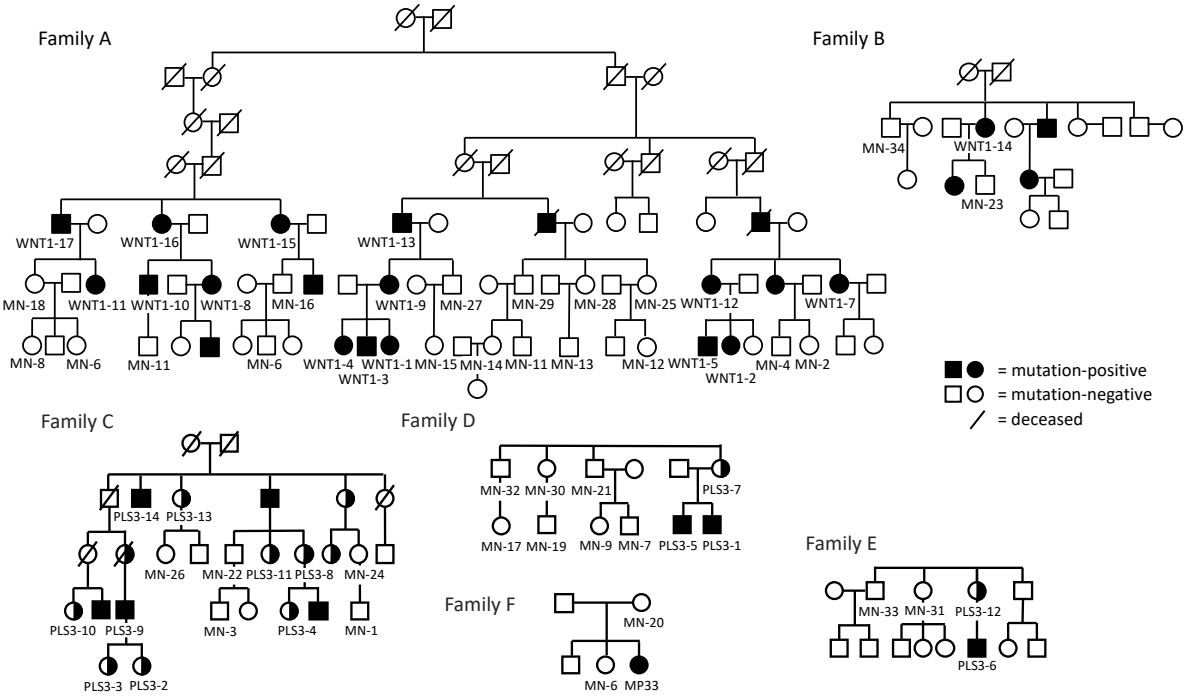

Family A
WNT1-17
WNT1-16
WNT1-15
WNT1-13
MN-18
WNT1-11
WNT1-10
WNT1-8
MN-16
WNT1-9
MN-27
MN-29
MN-28
MN-25
WNT1-12
WNT1-7
MN-8
MN-6
MN-11
MN-6
WNT1-4
WNT1-1
WNT1-3
MN-15
MN-14
MN-11
MN-13
MN-12
WNT1-5
WNT1-2
MN-4
MN-2
Family B
MN-34
WNT1-14
MN-23
= mutation-positive
= mutation-negative
= deceased
Family C
PLS3-14
PLS3-13
MN-26
MN-22
PLS3-11
PLS3-8
MN-24
PLS3-10
PLS3-9
MN-3
PLS3-4
MN-1
PLS3-3
PLS3-2
Family D
MN-32
MN-30
MN-21
PLS3-7
MN-17
MN-19
MN-9
MN-7
PLS3-5
PLS3-1
Family E
MN-33
MN-31
PLS3-12
PLS3-6
Family F
MN-20
MN-6
MP33

Figure 2.

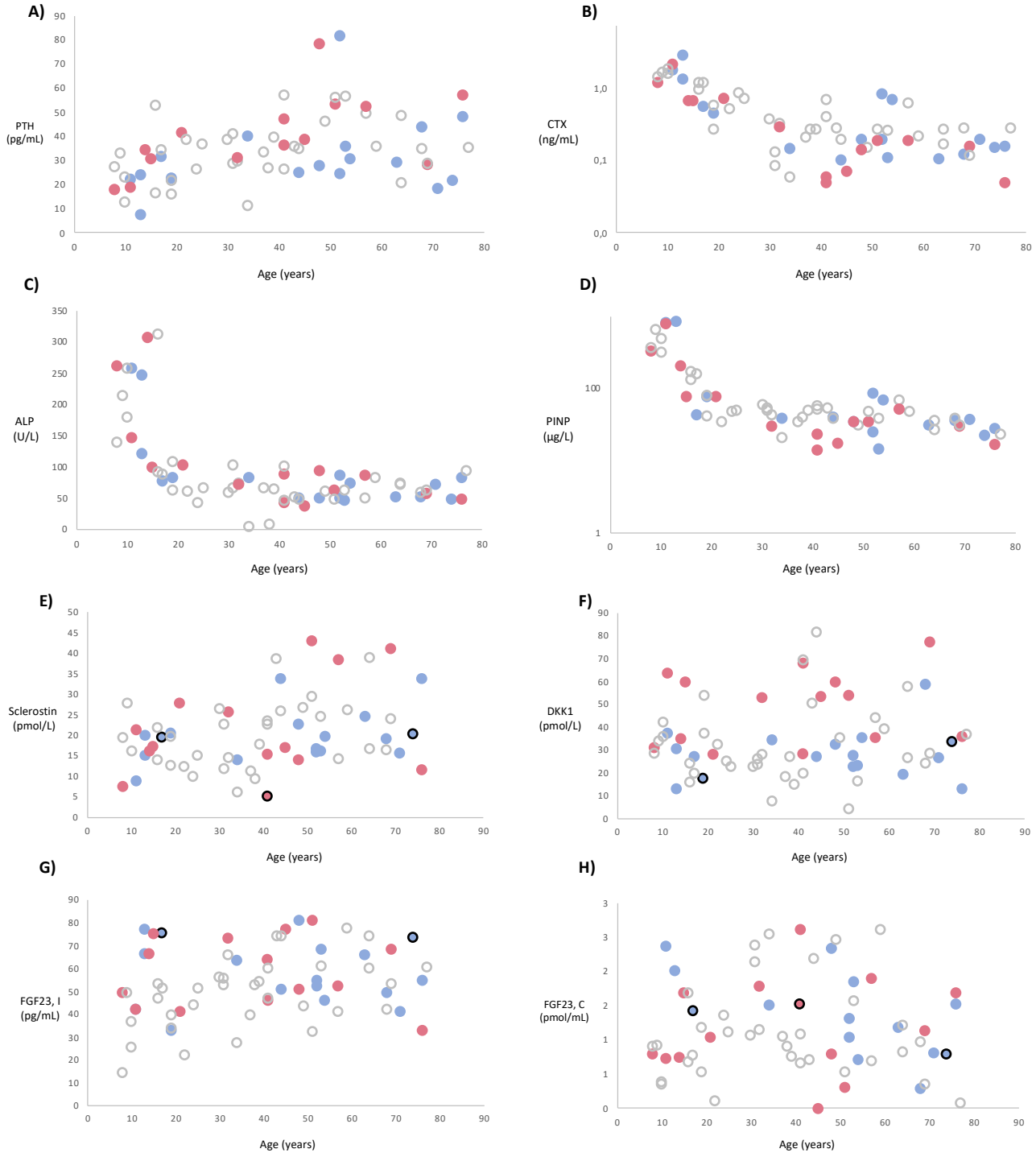

A)
PTH (pg/mL)
Age (years)
B)
CTX (ng/mL)
Age (years)
C)
ALP (U/L)
Age (years)
D)
PINP (µg/L)
Age (years)
E)
Sclerostin (pmol/L)
Age (years)
F)
DKK1 (pmol/L)
Age (years)
G)
FGF23, I (pg/mL)
Age (years)
H)
FGF23, C (pmol/mL)
Age (years)

Figure 3.

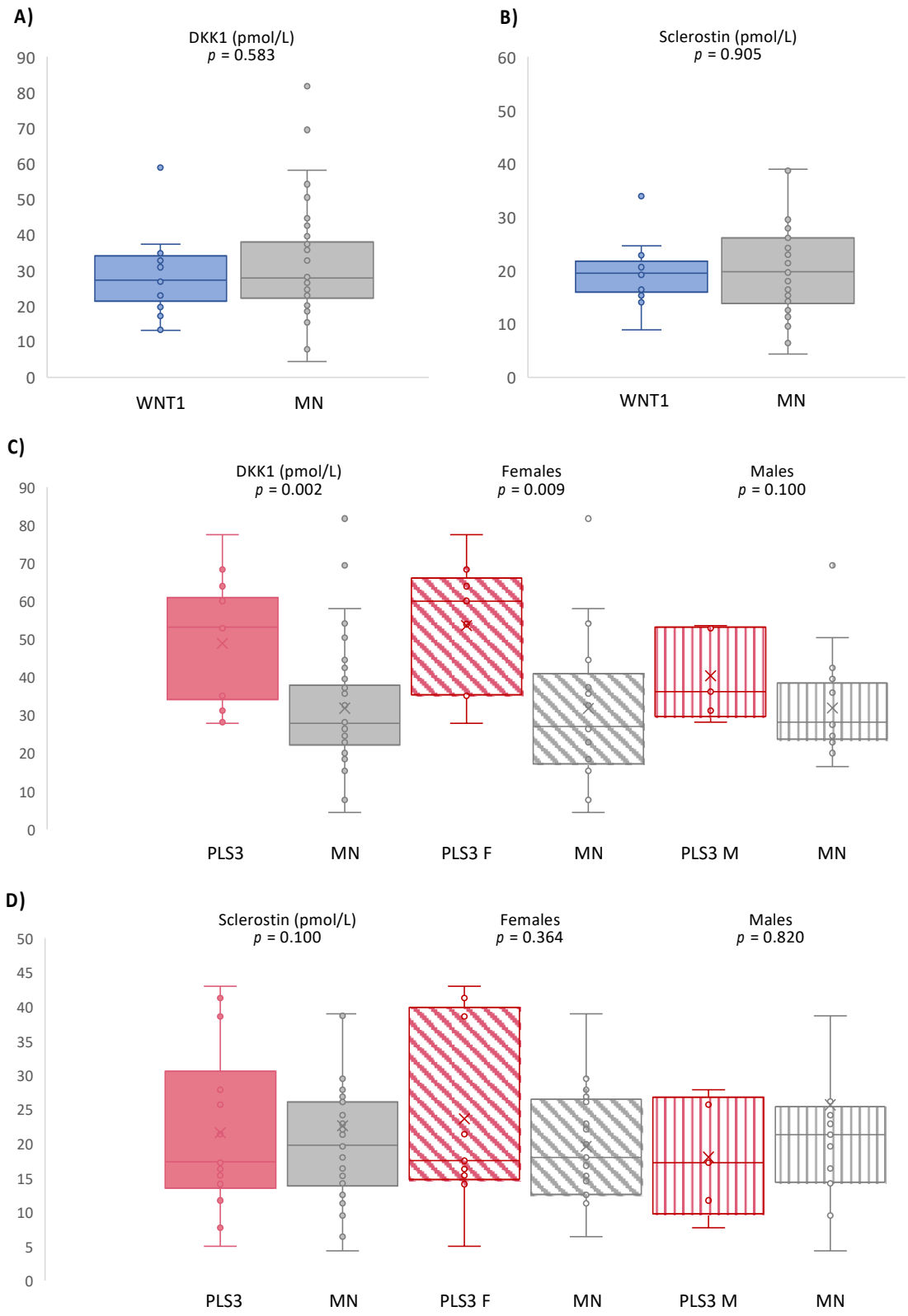
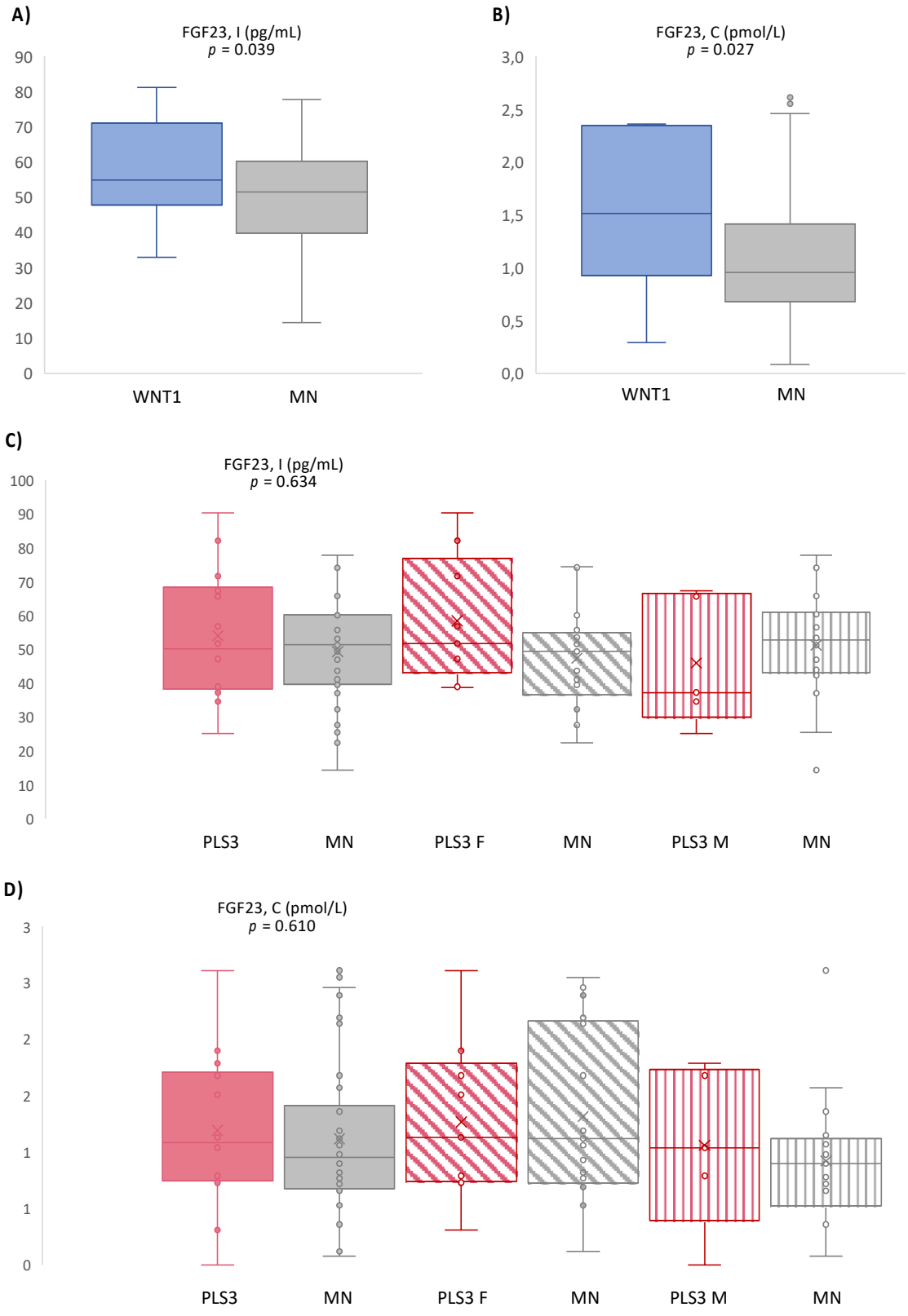

A)
DKK1 (pmol/L)
p = 0.583
90
80
70
60
50
40
30
20
10
0
WNT1
MN
B)
Sclerostin (pmol/L)
p = 0.905
60
50
40
30
20
10
0
WNT1
MN
C)
DKK1 (pmol/L)
p = 0.002
Females
p = 0.009
Males
p = 0.100
90
80
70
60
50
40
30
20
10
0
PLS3
MN
PLS3 F
MN
PLS3 M
MN
D)
Sclerostin (pmol/L)
p = 0.100
Females
p = 0.364
Males
p = 0.820
50
45
40
35
30
25
20
15
10
5
0
PLS3
MN
PLS3 F
MN
PLS3 M
MN

Figure 4.


A)
FGF23, I (pg/mL)
p = 0.039
90
80
70
60
50
40
30
20
10
0
WNT1
MN
B)
FGF23, C (pmol/L)
p = 0.027
3,0
2,5
2,0
1,5
1,0
0,5
0,0
WNT1
MN
C)
FGF23, I (pg/mL)
p = 0.634
100
90
80
70
60
50
40
30
20
10
0
PLS3
MN
PLS3 F
MN
PLS3 M
MN
D)
FGF23, C (pmol/L)
p = 0.610
3
3
2
2
1
1
0
PLS3
MN
PLS3 F
MN
PLS3 M
MN

Supplemental data

Supplemental Table 1. Clinical data for the 34 mutation-negative subjects.

Subject, Family	Gender	Age (years)	Number of peripheral fractures	Vertebral compression fractures	Prior osteoporosis medication, years since last dose	Inhaled glucocorticoids*	Postmenopausal
MN-2, A	F	9	1	NA	No	No	No
MN-6, F	F	16	1	NA	No	No	No
MN-8, A	F	19	5	NA	No	Yes	No
MN-9, D	F	19	1	NA	No	Yes	No
MN-10, C	F	22	2	NA	No	No	No
MN-12, A	F	25	6	NA	No	No	No
MN-14, A	F	31	2	NA	No	Yes	No
MN-15, A	F	31	2	NA	No	Yes	No
MN-17, D	F	34	0	NA	No	No	No
MN-18, A	F	37	0	NA	No	No	No
MN-20, F	F	39	8	NA	No	No	No
MN-24, C	F	44	0	NA	No	Yes	No
MN-25, A	F	49	0	NA	No	No	No
MN-26, D	F	51	1	NA	No	No	Yes
MN-28, A	F	57	0	NA	No	No	Yes
MN-30, D	F	64	2	NA	No	No	Yes
MN-31, E	F	64	0	NA	No	No	Yes
MN-1, C	M	8	1	NA	No	No	NA
MN-3, C	M	10	0	NA	No	No	NA
MN-4, A	M	10	2	NA	No	No	NA
MN-5, A	M	16	0	NA	No	No	NA
MN-7, D	M	17	0	NA	No	No	NA
MN-11, A	M	24	9	NA	No	No	NA
MN-13, A	M	30	4	NA	No	No	NA
MN-16, A	M	32	3	NA	No	No	NA
MN-19, D	M	38	0	NA	No	Yes	NA
MN-21, D	M	41	3	Yes	Yes, 5	No	NA
MN-22, D	M	41	1	NA	No	No	NA

MN-23, B	M	43	0	NA	No	No	NA
MN-27, A	M	53	0	Yes	No	No	NA
MN-29, A	M	59	1	NA	No	No	NA
MN-32, D	M	68	0	NA	No	No	NA
MN-33, E	M	69	0	Yes	Yes, 11	No	NA
MN-34, B	M	77	0	NA	No	No	NA

* Inhaled glucocorticoids with low to moderate dose; none of the subjects received oral glucocorticoids

Supplemental Table 2. Biochemical findings in 17 *WNT1* and 14 *PLS3* mutation-positive subjects and 34 mutation-negative subjects.

Subject, Family	Gender, Age (yrs)	S-Ca-Ion (mmol/L)	P-Pi (mmol/L)	S-25OHD (nmol/L)	1,25OH_2D (pmol/L)	S-PTH (pg/mL)	P-ALP (U/L)	P-CTX (ng/mL)	S-PINP (µg/L)	S-FGF23		S-DKK1 (pmol/L)	S-Sclerostin (pmol/L)	U-Pi/U-Crea (mmol/mmol)
										Intact (pg/mL)	C-terminal (pmol/L)			
***WNT1* mutation-positive subjects**														
WNT1-1, A	F, 11	1.26	1.26	90	173	22	258	1.79	820.00	42.0	2.36	37.4	8.9	1.82
WNT1-2, A	F, 13	1.32	1.32	75	140	24	122	1.35	N/A	66.4	11.02	13.2	20.2	0.99
WNT1-3, A	M, 13	1.25	1.25	112	94	**8**	247	2.95	852.00	77.3	2.01	30.9	15.3	1.90
WNT1-4, A	F, 17	1.25	1.25	116	96	31	77	0.56	43.40	75.5	1.42	27.3	19.5	2.51
WNT1-5, A	M, 19	1.27	1.27	**31**	168	22	83	0.45	76.10	32.9	6.17	17.2	20.6	2.33
WNT1-6, A	F, 34	1.24	1.24	105	**45**	40	84	0.15	38.21	63.8	1.51	34.8	14.0	15.09
WNT1-7, A	F, 44	1.32	1.32	82	67	25	51	0.10	39.79	51.0	5.71	27.2	33.9	1.30
WNT1-8, A	F, 48	1.22	1.22	125	98	28	51	0.20	34.59	81.2	2.33	32.7	22.8	1.10
WNT1-9, A	F, 52	1.44	1.44	64	127	82	86	0.84	85.26	52.3	1.04	22.9	16.0	2.41
WNT1-10, A	M, 52	1.24	1.24	74	172	24	50	0.20	25.01	54.7	1.31	27.7	16.9	1.60
WNT1-11, A	F, 53	1.21	1.21	102	103	36	47	0.11	14.69	68.7	1.85	23.5	16.4	1.10
WNT1-12, A	F, 54	1.22	1.22	136	123	31	74	0.71	68.92	46.1	0.71	35.8	19.9	2.25
WNT1-13, A	M, 63	1.24	1.24	146	114	29	52	0.11	30.74	66.1	1.18	19.7	24.6	1.17
WNT1-14, B	F, 68	1.20	1.20	80	119	44	52	0.12	36.47	49.5	0.29	58.9	19.2	1.30
WNT1-15, A	F, 71	1.24	1.24	88	124	18	72	0.20	37.79	41.2	0.81	26.8	15.8	2.05
WNT1-16, A	F, 74	1.25	1.25	97	72	22	48	0.15	22.16	73.4	0.78	33.5	20.1	1.15
WNT1-17, A	M, 76	1.28	1.28	89	88	48	84	0.16	28.30	54.9	1.52	13.3	34.0	0.96
***PLS3* mutation-positive subjects**														
PLS3-1, D	M, 8	1.34	1.43	77	162	18	262	1.21	332.00	34.6	0.79	31.3	7.7	1.18
PLS3-2, C	F, 11	1.24	1.13	61	189	19	146	2.20	796.00	51.8	0.73	64.0	21.4	1.62
PLS3-3, C	F, 14	1.26	1.45	61	115	34	307	0.69	206.80	47.2	0.75	35.2	16.3	0.84
PLS3-4, C	F, 15	1.26	1.31	**34**	186	31	100	0.69	75.82	56.8	1.68	60.1	17.5	N/A
PLS3-5, D	M, 21	1.27	1.39	55	185	41	103	0.73	76.71	37.3	1.04	28.2	27.9	2.20
PLS3-6, E	M, 32	1.25	1.03	69	138	31	73	0.29	30.20	65.6	1.79	53.0	25.7	1.73
PLS3-7, D	F, 41	1.27	0.89	**46**	91	36	89	0.06	23.22	48.6	1.51	28.0	5.0	1.70

PLS3-8, C	F, 41	1.22	0.84	**43**	185	47	43	0.05	14.20	38.7	2.61	68.4	15.4	0.72
PLS3-9, C	M, 45	1.21	1.02	90	156	39	37	0.07	17.12	25.2	0.00	53.6	17.2	2.01
PLS3-10, C	F, 48	1.16	0.85	61	206	78	94	0.14	35.02	39.0	0.79	60.1	14.1	1.81
PLS3-11, C	F, 51	1.19	0.80	85	158	54	64	0.19	34.53	82.2	0.31	54.0	43.0	1.50
PLS3-12, E	F, 57	1.21	1.09	95	109	52	87	0.19	51.82	90.3	1.90	35.7	38.6	0.99
PLS3-13, C	F, 69	1.23	1.05	131	114	28	58	0.16	30.12	71.6	1.13	77.6	41.3	1.66
PLS3-14, C	M, 76	1.21	0.84	69	108	57	49	0.05	17.00	67.4	1.68	36.3	11.7	1.69
Mutation-negative subjects														
MN-1, C	M, 8	1.24	1.42	74	110	27	139	1.46	360.00	14.4	0.90	28.9	19.6	4.25
MN-2, A	F, 9	1.24	1.59	68	165	33	214	1.69	660.00	49.5	0.93	34.1	27.9	N/A
MN-3, C	M, 10	1.25	1.42	59	154	23	179	1.91	490.00	25.5	0.36	36.0	16.4	4.31
MN-4, A	M, 10	1.28	1.55	70	192	13	257	1.64	312.00	37.0	0.39	42.5	21.3	1.15
MN-5, A	M, 16	1.32	1.42	**43**	157	16	313	1.22	171.00	47.0	0.68	24.6	14.2	1.59
MN-6, F	F, 16	1.20	1.41	69	93	53	93	0.96	130.33	53.6	1.68	16.0	22.1	1.25
MN-7, D	M, 17	1.26	1.22	73	118	35	89	1.21	157.83	51.3	0.78	20.2	4.3	3.16
MN-8, A	F, 19	1.27	1.56	69	100	16	108	0.27	41.67	39.8	1.19	37.4	12.7	1.83
MN-9, D	F, 19	1.29	0.91	60	135	22	63	0.59	79.12	33.7	0.53	54.2	19.9	2.14
MN-10, C	F, 22	1.19	0.99	**49**	235	39	61	0.53	34.02	22.4	0.12	32.7	12.5	1.95
MN-11, A	M, 24	1.30	1.19	**39**	144	26	44	0.86	48.07	43.9	1.36	25.4	10.2	3.19
MN-12, A	F, 25	1.24	1.53	70	127	37	67	0.73	50.34	51.6	1.12	23.0	15.3	2.40
MN-13, A	M, 30	1.27	1.38	54	117	39	60	0.38	59.37	56.5	1.07	22.9	26.5	1.01
MN-14, A	F, 31	1.24	0.88	**36**	68	29	104	0.13	49.08	55.7	2.14	24.2	22.9	2.36
MN-15, A	F, 31	1.30	1.34	**26**	133	41	66	0.09	53.13	53.1	2.39	26.5	12.0	2.20
MN-16, A	M, 32	1.25	0.91	**41**	93	30	74	0.33	43.10	65.8	1.15	28.2	14.6	1.15
MN-17, D	F, 34	1.30	0.87	63	269	11	5	0.06	20.55	27.7	2.55	7.8	6.4	0.97
MN-18, A	F, 37	1.27	**0.51**	60	134	33	67	0.21	34.17	39.5	1.06	18.5	11.3	1.46
MN-19, D	M, 38	1.29	0.83	78	98	27	8	0.27	39.31	52.8	0.9	27.6	9.5	1.41
MN-20, F	F, 39	1.20	0.84	103	103	40	65	0.27	48.89	54.4	0.77	15.4	18.0	1.10
MN-21, D	M, 41	1.23	0.98	84	87	27	46	0.41	58.46	47.0	1.09	69.5	23.6	1.73
MN-22, D	M, 41	1.23	1.05	144	144	57	102	0.71	51.22	60.4	0.66	20.1	22.9	1.51
MN-23, B	M, 43	1.27	1.13	80	74	36	52	0.28	52.74	74.2	0.72	50.5	38.7	1.54
MN-24, C	F, 44	1.18	0.87	77	89	35	49	0.20	38.72	74.2	2.19	81.8	26.1	2.48

MN-25, A	F, 49	1.22	0.84	90	83	46	61	0.15	31.21	43.7	2.46	35.7	26.9	1.24
MN-26, D	F, 51	1.16	1.27	65	93	56	48	0.27	47.99	32.3	0.53	4.4	29.5	2.75
MN-27, A	M, 53	1.22	1.05	73	57	56	63	0.27	38.77	61.2	1.57	16.6	24.6	2.32
MN-28, A	F, 57	1.28	**1.09**	**39**	109	50	50	0.62	70.13	41.2	0.69	44.6	14.5	2.67
MN-29, A	M, 59	1.26	1.08	**21**	71	31	83	0.22	47.42	77.8	2.61	39.6	26.2	2.18
MN-30, D	F, 64	1.28	1.25	73	143	21	75	0.27	35.59	60.1	0.82	27.1	16.8	2.86
MN-31, E	F, 64	1.18	0.85	112	112	49	73	0.17	27.01	74.4	1.21	58.1	39.0	1.76
MN-32, D	M, 68	1.30	0.79	74	122	35	59	0.28	38.74	42.3	0.98	24.7	16.5	N/A
MN-33, E	M, 69	1.16	0.84	89	119	29	64	0.12	31.49	53.4	0.36	28.8	24.2	1.80
MN-34, B	M, 77	1.28	1.02	87	64	36	94	0.29	23.01	60.9	0.08	37.3	123.0	0.87

F = female, M = male, N/A = not available. Supranormal values are underlined and subnormal values are in **bold**. Normal ranges according to HUSLAB Laboratory (females/males): S-Ca-ion (serum ionized calcium) 1.16-1.3; P-Pi (phosphate) 2–12 years 1.2–1.8, 13–16 years 1.1–1.8, 17 years 0.8–1.4, females >18 years 0.76–1.41, males 18–49 years 0.71–1.53, males >50 years 0.71–1.23; S-25OHD (vitamin D, nmol/L) >50; normal ranges for D-1,25 according to United Medix Laboratories Ltd (1.25-dihydroxyvitamin D) 48–190; ALP (alkaline phosphatase) 8–9 years 115–345, 10–11 years 115–435/115–335, 12–13 years 90–335/125–405, 14–15 years 80–210/80–445, 16–18 years 35–125/55–330, >18 years 35–105. Normal ranges for PTH and CTX using the IDS-iSYS assay (Immunodiagnostic Systems, Ltd., Bolton, UK): PTH adults 11.5–78.4; CTX (collagen type 1 cross-linked C-telopeptide) pre-menopausal females 0.034–0.635, post-menopausal females 0.034–1.037, males 0.038–0.724. Normal ranges for PINP (type I procollagen intact N-terminal propeptide) according to Morovat et al., 2013[42] (females/males): 5–8 years 307–985/200–900, 9–12 years 386–1070/323–1242, 13–16 years 59.3–672/142–6929, 17–20 years 25.2–160/28.1–369, pre-menopausal women 13.7–71.1, post-menopausal women 8.2–82.6, men 18–45 years 19.4–95.4, men >45 years 12.8–71.9.